

DNA vaccination for the priming of neutralizing antibodies against non-immunogenic STa enterotoxin from enterotoxigenic *Escherichia coli*

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Abstract

In order to test the use of DNA vaccination for its capacity to induce antibodies against the non-immunogenic heat-stable enterotoxin STa from *Escherichia coli*, BALB/c mice were immunized with plasmid DNA encoding hybrid proteins made by the insertion of wild type STa or insertion of the Cys6Ala, Cys17Ala and Cys6Ala–Cys17Ala STa mutants at positions 195 or 216 of the TEM-1 β -lactamase. No STa specific antibodies could be detected after three plasmid injections, but a subsequent boost with native STa peptide was capable of inducing low levels of neutralizing antibodies, as tested in the suckling mouse assay. Highest STa specific responses were found in mice primed with the double mutated STa inserted in position 195. This plasmid induced highest T-cell responses to the TEM-1 protein, indicating that priming of helper T-cell responses to the carrier protein was essential. Mixed IgG1/IgG2a isotypes also reflected this T helper 1 type priming. Moreover, insertion into loop A of the TEM-1 carrier may be more suitable than insertion into loop B, because of reduced competition between carrier and hapten B cell responses.

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1. Introduction

In newborn calves and piglets, enterotoxigenic *Escherichia coli* (ETEC) is a major cause of enteric colibacillosis, characterized by a profuse fluid diarrhoea leading to dehydration and even death [1,2]. ETEC are also responsible for diarrhoea in children of and travellers to developing countries [3]. The fluid secretion at the basis of the diarrhoea is induced by two main classes of toxins active on the enterocytes, hence named enterotoxins: the heat-labile enterotoxin (LT) and the heat-stable enterotoxin (ST) [4]. Moreover, two subtypes of ST enterotoxins have been described, STa and STb, which differ in primary structures and biological activities [5]. The STa enterotoxins are further subdivided into two

genotypes, which differ slightly in their primary structures, but not in their biological activities: the STa1 (or STaP, because first recognised in a porcine ETEC) and the STa2 (or STaH, because first recognised in a human ETEC). STa1 and STa2 are composed of 18 and 19 amino acids, respectively [6]. Bovine ETEC typically produce the STa enterotoxin subtype and the only genotype identified is STa1 [7–10]. Both STa enterotoxins comprise six cysteines which form three intramolecular disulfide bridges that are necessary for the toxin activity [11].

Because of its small size, STa is poorly antigenic and not immunogenic in its native form. However, STa can be made immunogenic when coupled to an appropriate carrier in a standard hapten-carrier configuration. Several approaches have been explored to obtain non-toxic immunogenic molecules for safe vaccine design. Several proteins have been coupled chemically to STa in order to increase the

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immunogenicity of the enterotoxin, including bovine serum albumin [12], the B-subunit of cholera toxin (CTB) [13], and the heat-labile enterotoxin [13,14]. Some hybrid fusion proteins have been constructed between STa and the major protein subunit ClpG of *E. coli* CS31A surface antigen (ClpG-ST) [15], between STa and one or more subunits of the cholera toxin [16,17] and between STa and LT [18,19]. The OmpC outer-membrane protein and the flagellin have also been used as a carrier for ST [20,21]. In general, these constructions either failed to elicit neutralizing antibodies or retained some degree of STa-associated toxicity, suggesting that the immunogenic properties of the toxin are influenced by conformation associated with toxicity.

Ideally, vaccines should be not only safe and effective but also simple and inexpensive to manufacture. This is particularly true for veterinary vaccines, but also for vaccines needed in developing countries. The production and purification of hybrid protein vaccines is an intensive and expensive labour. DNA vaccines are stable, easy to produce, and newly identified antigens can be rapidly incorporated. DNA immunization is a potent method for inducing both humoral and cell-mediated immune response [22–24]. Moreover, immune responses induced by DNA vaccines may display a broader epitopic repertoire than immune responses induced by traditional peptide or protein vaccines [25–27].

In this study, we have used the polynucleotide vaccination approach, to genetically insert the nucleotide sequence coding for the mature form of the STa1 toxin into the *amp^R* gene encoding the TEM-1 β -lactamase. This enzyme is a 263-amino-acid protein that hydrolyses the β -lactam ring of antibiotics and renders bacteria resistant [28,29] to penicillins and related antibiotics. The sequence encoding the STa oligopeptide was genetically inserted into the TEM-1 gene within sequences corresponding to two different surface loops of the β -lactamase, in position 195 and 216, respectively, named loop A and loop B. Three mutated STa sequences in which one or two cysteine residues were replaced by alanines, were also inserted in the TEM-1 molecule at position 195. These mutations disrupt one or two disulfide bridges and normally cause a complete loss of toxicity [16,30]. The hybrid genes were cloned into an appropriate plasmid DNA expression vector. The plasmids were tested for their capacity to induce a TEM-1 and STa specific immune response in BALB/c mice. In a second phase, an attempt was made to increase the STa specific antibody production, by boosting the DNA immunized mice with synthetic peptide.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

E. coli JM109 (Promega Corporation, USA) was used. For plasmid production, bacteria were grown in luria-bertani (LB) broth at 37 °C during 6–8 h for the preculture and in super-broth (SB) at 18 °C during 12–18 h for the culture [31].

Medium was supplemented with 12.5 μ g/ml tetracycline for the culture of bacteria transformed with plasmid pFH and with 50 μ g/ml kanamycin for bacteria transformed with plasmid V1Jns.tPA. Two plasmids pFH, coding for TEM-1 with a unique *KpnI* restriction site (designed by H) in positions 195 and 216, respectively (pFH195H and pFH216H), were kindly provided by Dr B. Hallet [32]. These plasmids were used to construct the hybrid gene. The V1Jns.tPA vector [33] was used to construct six different DNA vaccines. The genes of interest were first subcloned in pGEM[®]-T easy vector (Promega, USA). Cultures were incubated overnight on ampicillin-containing agar plates. The addition of IPTG (isopropyl- β -D-thiogalactopyranoside, Eurogentec, Seraing, Belgium) and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, Roche Diagnostics, Brussels, Belgium) allowed the selection of colonies containing the insert (white colonies).

2.2. Plasmid construction

STa was inserted in positions 195 and 216 of TEM-1 β -lactamase. TEM195H, TEM195STa and TEM216STa encoding genes were amplified without their bacterial signal sequences from plasmid pFH by PCR using oligonucleotide “sense” (5′ GGAAGATCTCTCACCCAGAAACGCTGTGAAAGTA 3′) and “anti-sense” primers (5′ GGAA-GATCTAGTTGCCTGACTCCCCGTCGTGTA 3′) which contained a *BglII* restriction site. The PCR fragments were digested with *BglII*. They were isolated on a 1% agarose gel and extracted with “GFX[™] PCR DNA and Gel Band Purification Kit” (Amersham Pharmacia Biotech, UK). Fragments were, finally, ligated into the *BglII*-digested and dephosphorylated V1J-ns.tPA vector [33]. *E. coli* JM109 was used as host for the three plasmids V1J195H, V1J195STa, and V1J216STa. They were purified using “Maxiprep GFII[™] Endo-Free Kit” (Q-BIOgene), adjusted to a final concentration of 1 mg/ml and stored in sterile PBS (30 mM K₂HPO₄, 150 mM NaCl, pH 7.3) at –20 °C until immunization.

2.3. Substitutions of two STa's cysteines

Site-directed mutagenesis was performed on plasmid V1J195STa using the “QuickChange[™] Site-Directed Mutagenesis Kit”. The Cys6Ala mutation was made using oligonucleotides “OneA” (5′ TACCCAACACGTTTACT-GCGCCGAACCTTTGCTGCAACCCAGC 3′) and “OneB” (5′ GCTGGGTTGCAGCAAAGTTCGGCGCAGTAAAA-CGTGTTGGGTA 3′) and the Cys17Ala mutation was made with oligonucleotides “TwoA” (5′ CCAGCATGCGCAGGT-GCCTACGGGGTACCCC 3′) and “TwoB” (5′ GGGGTAC-CCCGTAGGCACCTGCGCATGCTGG 3′) (Eurogentec, Liège, Belgium). The double mutation (Cys6Ala–Cys17Ala) was made by two successive oligonucleotide-directed mutagenesis steps. The different mutations were confirmed by DNA sequencing by the method of Sanger et al. [34] with the help of an A.L.F. sequencer. We, finally, obtained three

plasmids with different hybrid genes: V1J195STa Cys6Ala, V1J195STa Cys17Ala and V1J195STa Cys6,17Ala. These mutant plasmids were introduced into *E. coli* JM109 by heat shock and kanamycin resistant transfected bacteria were selected, purified with “Maxiprep GFII™ Endo-Free Kit” (Q-BIOgene) and stored in filtered PBS at -20°C until use. The purity of the preparations was checked by measuring the A260/A280 ratio.

2.4. Transfection of eukaryotic cells and Western blot analysis

The ability of the six plasmids to express the encoded wild type TEM-1 or the hybrid TEM-STa proteins was tested by transfection into BHK-21 cells (Baby Hamster Kidney, Bio Whittaker, Europe) using Lipofectamine™ Reagent (Gibco-BRL, Gaithersburg, Md). The cells were grown in 24-well culture plates with DMEM (Gibco) containing 10% heat-inactivated foetal calf serum (FCS). Between 0.1 and 0.2 μg of plasmid DNA was used to transfect 2.4×10^5 cells per well. After 48 h, protein extracts were obtained by lysis in buffer containing 60 mM Tris-HCl pH 6.8, 10% glycerol, 1% sodium dodecyl sulphate (SDS) and β -mercaptoethanol. Cell lysates were then separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane (Millipore Corporation, Madison, USA). Incubation of membrane with primary antibody (rabbit polyclonal anti-TEM) was followed by incubation with alkaline phosphatase-conjugated anti-rabbit as secondary antibody (Bio-Rad, CA, USA). The primary and the secondary antibodies were diluted 1000- and 3000-fold, respectively, in Tris-buffered saline containing 1% (w/v) bovine serum albumin and 0.5% (v/v) Tween 20. For normalization, the same nitrocellulose membrane was also analyzed for detection of the constitutively produced housekeeping protein β -actin, using a mouse antibody against human β -actin (Gentaur, Brussels, Belgium) and a goat anti-mouse conjugated with alkaline phosphatase as a secondary antibody (Bio-Rad, CA, USA). The primary and the secondary antibodies were diluted 500- and 3000-fold, respectively. Positive protein bands were detected by using nitro blue tetrazolium (dNTB)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) chemistry (Roche Applied Science) as recommended by the manufacturer. NIH software ImageJ was used to analyze the intensity of Western blot bands. The normalised ratio was then obtained by comparing the TEM band's intensity to the corresponding β -actin band.

2.5. Immunization

Female BALB/c mice were anesthetized by intra-peritoneal injection of ketamine-xylazine (100 mg/kg–10 mg/kg) and injected three times, at 3-week intervals, in both quadriceps with $2 \times 50 \mu\text{g}$ of plasmid V1Jns.tPA encoding one of the six proteins, as previously described [35]. Subsequently,

Table 1

(A) Immunization scheme used in the seven mouse groups (five animals per group) and (B) Experimental schedule

(A)										
Group	Plasmid immunization	Protein expressed by the DNA plasmid		Boost STa						
1	pV1J195H	TEM195H		Yes						
2	pV1J216STa	TEM216STa		Yes						
3	pV1J195STa	TEM195STa		Yes						
4	pV1J195STa Cys6Ala	TEM195STa Cys6Ala		Yes						
5	pV1J195STa Cys17Ala	TEM195STa Cys17Ala		Yes						
6	pV1J195STa Cys6,17Ala	TEM195STa Cys6,17Ala		Yes						
7	No	–		Yes						

(B)										
	Days									
	0	21	42	63	78	91	113	131	147	
Plasmid immunizations	X	X	X							
Peptide boost					X		X			
Bleeding	X			X		X	X	X	X	
Antibody measurement:				X						
IgG1–IgG2a against TEM-1										
Antibody measurement:										X
IgG1–IgG2a against STa										
IFN- γ production										X
TEM-1 enzymatic assay				X						
STa neutralization										X

In (B), days of immunization, peptide boost, bleeding, antibody and IFN- γ measurements, enzymatic and neutralization assays are represented by a cross.

two intramuscular boosts were given with 10 μg of synthetic STa peptide (Eurogentec, Liège, Belgium) in MPL-A-QS21 on days 78 and 113. Sera were collected on days 0, 63, 91, 113, 131 and 147 by retro-orbital bleeding and stored at -20°C until use. The experimental schedule and the different experimental groups are indicated in Table 1.

2.6. Antibody measurement: IgG1 and IgG2a isotypes

TEM-1 and STa specific antibodies were detected by ELISA. Ninety-six-well microtiter plates (Maxisorp Nunc-Immuno Plate, Denmark) were coated with 250 ng/50 μl of β -lactamase (TEM195H) per well for the detection of antibodies against the carrier TEM-1, and with 400 ng/50 μl of synthetic peptide STa per well for the detection of antibodies against the STa toxin. After washing, 100 μl of blocking buffer (casein hydrolysate) was added to each well and plates were incubated at 37°C for 30 min. After washing with PBS containing 0.05% Tween 20, serial two-fold dilutions (starting at 1:50) of sera in blocking buffer were added and incubated for 1 h at 37°C . The bound antibodies were detected by 1-h incubation at 37°C with horseradish peroxidase (HRP)-labelled sheep anti-mouse IgG1 or IgG2a (Sheep Anti Mouse IgG1 Affinity Purified-HRP, Sheep Anti Mouse IgG2a Affinity Purified-HRP, The Binding Site, Birmingham, UK). The reaction was developed using tetramethylbenzidine (TMB) during 10 min, the enzyme reaction was stopped by addition

of 1M H₃PO₄ and the absorbance was read at 450 nm. Titres were determined as the log₂ value of the highest serum dilution giving an absorbance higher than the mean absorbance value of pre-immune sera \pm 3 S.D. Titres were given as the arithmetic mean \pm S.E.

2.7. Spleen cell cytokine production [36]

On day 147 (34 days after the second peptide injection), mice were killed by cervical dislocation and spleens were removed aseptically. Spleens from individual mice were homogenized in a loosely fitting Dounce homogenizer, spleen cells were adjusted to a concentration of 4×10^6 leukocytes/ml and cultured in round-bottom microwell plates (Nunc) in RPMI 1640 medium supplemented with glutamine, HEPES, 2-mercaptoethanol, antibiotics and 10% heat-inactivated FCS. Twenty microliters of the different antigens (TEM-1 protein (3 and 10 μ g/ml final), STa synthetic peptide (10 μ g/ml final) the T-cell mitogens concanavalinA (ConA, Pharmacia, 4 μ g/ml) and pokeweed mitogen (PWM, Sigma, 5 μ g/ml) were mixed with 180 μ l of cell suspension as described before [37]. Cell cultures were

incubated at 37 °C in a humidified CO₂ incubator, pooled supernatants from three wells were harvested after 72 h and stored frozen at –20 °C until assay.

2.8. IFN- γ assay

IFN- γ content in 72 h culture supernatant was determined by using EIA kit: “CytElisaTM Mouse IFN- γ ” (CYTimmune Sciences Inc, Maryland). Results are expressed in pg/ml.

2.9. Enzymatic β -lactamase assay

The β -lactamase neutralizing capacity of TEM-1 specific serum antibodies was determined by pre-incubating the TEM-1 enzyme (2 μ g/ml) with two- and five-fold dilutions of the different sera in a volume of 25 μ l for 30 min and 1 h at room temperature. Serum dilutions were made in PBS (50 mM, pH 7) containing 0.2 mg bovine serum albumin/ml. The β -lactamase activity of TEM-1 was measured as the initial rate of nitrocefin hydrolysis. The reaction was followed at 482 nm ($\Delta\epsilon = 15,000 \text{ M}^{-1} \text{ cm}^{-1}$) with the help of Uvikon 860 spectrophotometer.

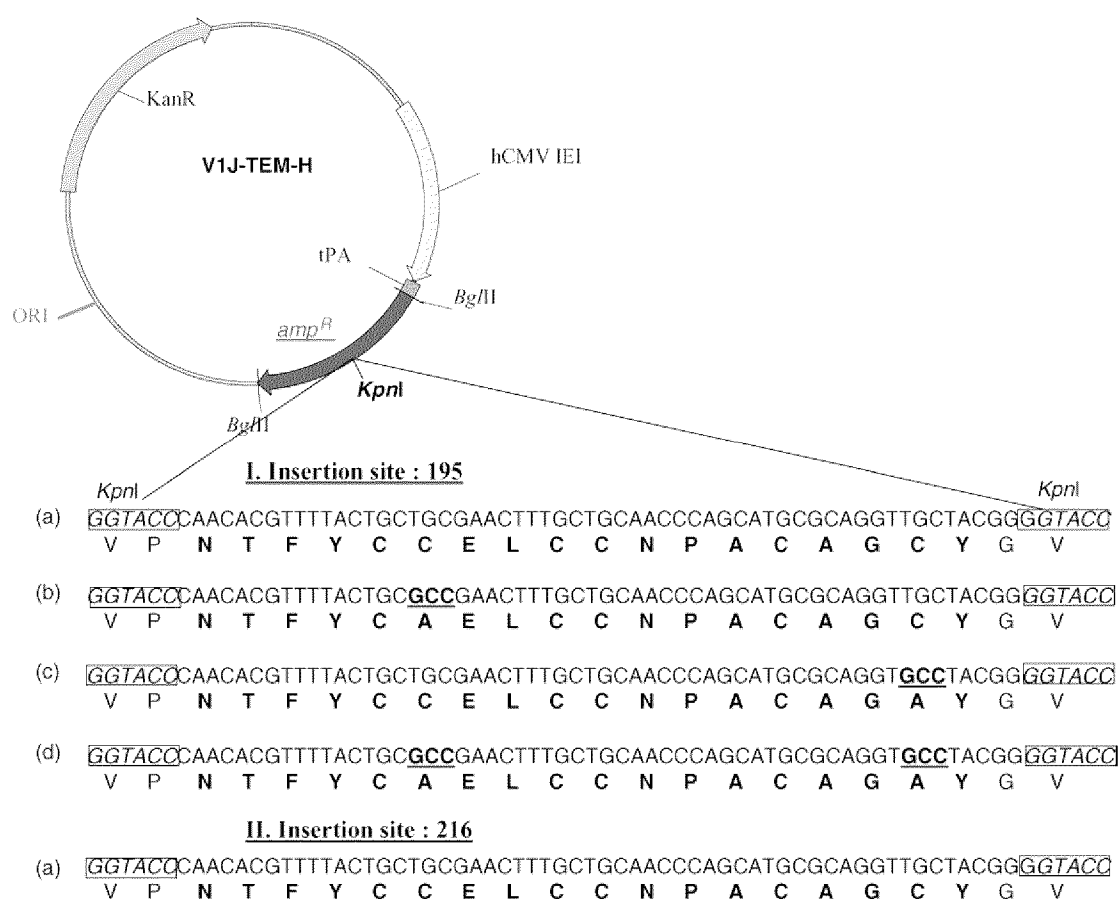


Fig. 1. Schematic representation of the various plasmid constructs. The genes coding for TEM195H, TEM216STa, TEM195STa, TEM195STa Cys6Ala, TEM195STa Cys17Ala and TEM195STa Cys6,17Ala were amplified by PCR and inserted in the Bg/II restriction site of the V1J.ns-tPA vector. The sequences coding for the native enterotoxin STa (a); the mutated Cys6Ala enterotoxin (b); the mutated Cys17Ala enterotoxin (c); and the mutated Cys6, 17Ala enterotoxin (d) are represented with their respective amino acid sequences below. These sequences were introduced in positions 195 (I) and 216 (II) of the TEM-1 protein.

2.10. Antibody neutralisation of STa studied by suckling mouse assay

The toxicity of STa can only be measured by the so-called suckling mouse assay, i.e. the measurement of fluid secretion into the intestinal lumen of newborn mice [38] (protocol accepted by the Ethical Committee of the University of Liège, 26 April 2000, Protocol 86). To test the neutralizing activity of anti-STa sera on this biological activity, a predetermined active dose of STa was incubated with a 1:8 dilution of anti-STa sera pooled from groups of immunised mice. This active dose corresponds to the highest dilution of the semi-purified STa giving a positive response. In each group, only sera from immunised mice that showed positive STa antibody titres in ELISA were pooled. The dilutions were made in 0.7 ml PBS buffer and incubated for 16 h with shaking at 4 °C. Each newborn mouse received 0.1 ml of the appropriate sample using a one ml syringe with a needle. After 3 h at room temperature, the animals were sacrificed and the ratio of gut weight to remaining carcass weight was measured. A gut/carcass ratio ≥ 0.085 or higher was considered as positive. For sample 6, pooled serum was further tested at dilution 1:16 and 1:32 of the anti-STa serum. The positive control was the highest dilution of the semi-purified STa (from ETEC strain 1676 [39]) giving a positive response and the negative control was the supernatant of an overnight broth culture of strain HS [40].

2.11. Statistics

Statistical analyses were performed using the Mann–Whitney *U*-test (non parametric test), for comparison of two groups, groups 6 and 7. Values of $P < 0.05$ were considered as significant.

3. Results

3.1. Construction of the various hybrid plasmids

The wild type STa gene was inserted in positions 195 and 216 of the TEM-1 β -lactamase. The three mutated STa genes (in which one or two cysteine residues were replaced by alanines) were inserted in position 195. Table 1 shows the immunization scheme and the proteins encoded by the different plasmids. Fig. 1 shows a schematic description of the different constructions.

3.2. Expression of the hybrid proteins in transiently transfected BHK-21 cells

The ability of the different plasmids to express the hybrid proteins was evaluated in transiently transfected BHK-21 cells. Since the TEM-1 β -lactamase and the heat-stable enterotoxin STa originate from prokaryotic organisms and since the production of the hybrid TEM-STa is not easy

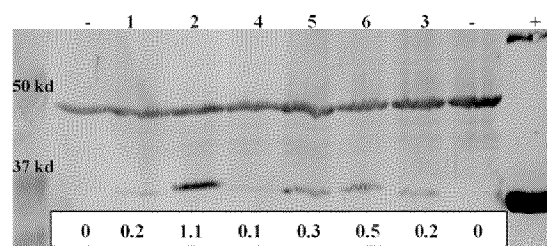


Fig. 2. Expression of the TEM hybrid proteins by the different plasmids. Lysates of transfected BHK-21 cells were analysed by Western blot using rabbit anti-TEM sera and mouse anti- β -actin antibodies. Cells were transfected with plasmid (1) VJ195H, (2) VJ216STa, (4) VJ195STa Cys6Ala, (5) VJ195STa Cys17Ala, (6) VJ195STa Cys6,17Ala, (3) VJ195STa. Lysates of non transfected cells (–) and purified 195H protein (+) were used as negative and positive controls for the TEM expression, respectively. The protein ladder used in this assay was “Precision Plus Protein™ Standard” (Bio-Rad). The levels of protein expression are represented (boxed) and were obtained by comparing the intensities of the TEM bands to that of the β -actin internal control (42 kDa).

to obtain, it was necessary to verify this expression in eukaryotic cells before starting the in vivo immunizations. TEM-1 carrier protein could be detected in cells transfected with all plasmids. Expression levels of TEM-1 were normalized to those of the house keeping protein β -actin in Western blot using TEM-1 and β -actin specific antibodies. This indicated that plasmids 2 and 6 were expressed two- to five-fold better than the other plasmids (Fig. 2).

3.3. Antibody production against the carrier protein TEM-1 in mice vaccinated with DNA encoding the various plasmid constructs

A positive anti- β -lactamase antibody response was observed 3 weeks after the third DNA injection. Except for mice vaccinated with the plasmid pVJ195STaCys17 (group 5), vaccination with all plasmids induced IgG1 (Fig. 3a) and IgG2a (Fig. 3c) antibodies against TEM-1. Despite three immunizations, humoral responses showed some degree of individual variation and in each group of five mice, some failed to show detectable antibody titres, especially of the IgG1 isotype. Confirming previous findings with another VJJ.ns-tPA based DNA vaccine encoding Ag85A from *Mycobacterium tuberculosis* [41], TEM-1 specific IgG2a titres were higher than those of IgG1 in the sera of these intramuscularly vaccinated BALB/c mice.

3.4. Antibody production against the STa enterotoxin in mice vaccinated with DNA encoding the various plasmid constructs and boosted with synthetic STa peptide

No STa specific IgG could be detected after three DNA immunizations. As DNA vaccines are well known for their priming capacities [42], we decided to boost the DNA vaccinated mice with the oligopeptide toxin, which is not immunogenic in its native form. Following two intramuscular

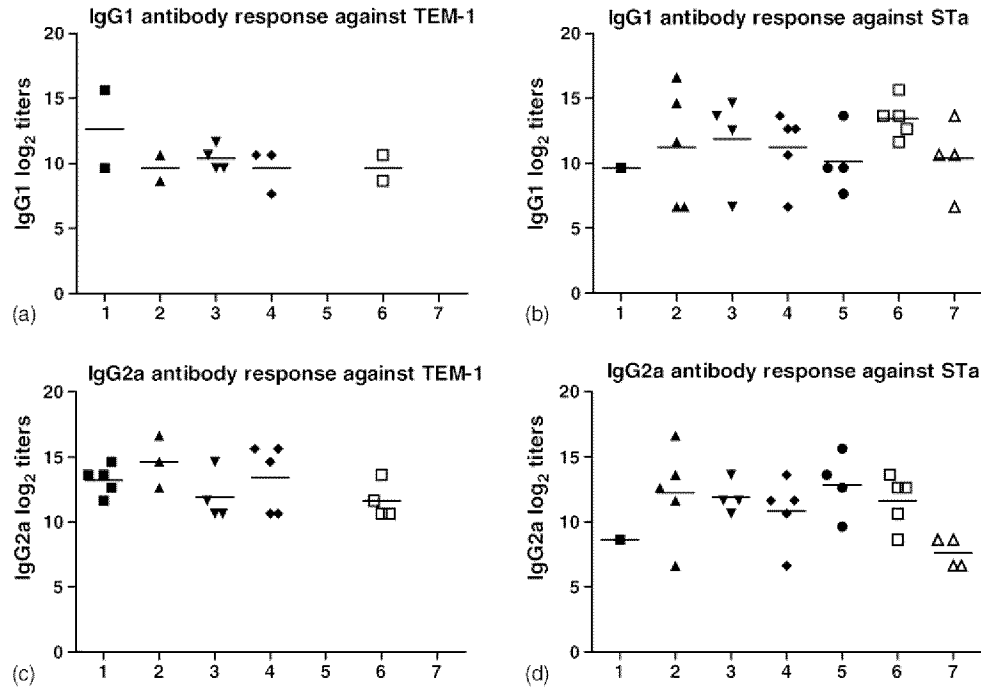


Fig. 3. Antibody production in response to the carrier protein TEM (a and c) and to the STa enterotoxin (b and d). BALB/c mice were vaccinated with plasmid DNA encoding TEM (group 1), TEM216STa (group 2), TEM195STa (group 3), TEM195STa Cys6Ala (group 4), TEM195STa Cys17Ala (group 5), TEM195STa Cys6,17Ala (group 6), or did not receive any plasmid (group 7) and boosted with two injections of STa peptide. Each group was composed of five animals. Sera from mice were collected individually on day 63 (3 weeks after the third DNA immunization) and 147 for the detection of antibody response, respectively, against TEM and the STa enterotoxin. IgG1 and IgG2a antibody response against the carrier and the enterotoxin were shown on graphs a–d. Only mice that showed a positive antibody response were represented on this graph. Data represent the log₂ antibody titres, determined as the highest serum dilution giving an absorbance higher than the mean absorbance of five pre-immune sera ± 3 S.D. Horizontal bars represent the mean of each group.

STa injections, mice which had been initially primed with plasmid encoding hybrid TEM-STa genes showed high STa specific antibody titres (Fig. 3b and d). In contrast, no STa specific serum IgG1 or IgG2a could be detected in four out of five mice immunised with the vector encoding only the TEM-1 carrier protein. These findings clearly indicate that STa had been successfully expressed in the TEM-STa hybrid proteins and that a STa specific priming had occurred in the DNA vaccinated mice. Interestingly, and somewhat unexpectedly, enterotoxin specific antibodies were also detected in mice that only received the two intramuscular STa peptide injections (group 7). However, whereas IgG1 antibody titres in group 7 were similar to those in the DNA primed groups, (except for group 6 which was significantly higher), the IgG2a titres in group 7 mice were clearly lower (Fig. 3d), again indicating that the DNA vaccinated mice had indeed been primed against the STa enterotoxin. More IgG2a antibodies were produced by priming with pV1J195STa Cys6,17Ala (group 6) than by peptide immunization only (group 7) ($P < 0.05$). Two mice died before the end of the experiment on day 113 (group 3) and 147 (group 5).

3.5. IFN- γ production in spleen

Mice were sacrificed on day 147, 5 weeks after the second STa injection and spleen cells from individual mice were

analyzed for the production of IFN- γ in response to the TEM-1 protein (Fig. 4a) and the STa peptide (Fig. 4b). As positive controls, cells were also stimulated with the polyclonal mitogens ConA and PWM. As for antibody production, a considerable variation was observed from one animal to another (Fig. 4a and b). Therefore, mean responses were also calculated on positive animals only, using cut-off levels for positivity based on mean values obtained in mice from group 7 for anti-TEM-1 responses and of group 1 for anti-STa responses (Fig. 4c and d). Positive IFN- γ responses against TEM-1 were detected following immunization with all plasmids, but responses were lower in groups 4 and 5, vaccinated with the single mutated STa and higher in groups 2 and 3, vaccinated with the wild type STa toxin. Finally, IFN- γ titers were highest in spleen cell cultures from mice of group 6, immunised with the doubly mutated STa inserted into loop A. Similar differences were observed following stimulation with the lower dose of TEM-1 at 3 $\mu\text{g/ml}$ (data not shown). IFN- γ responses against the STa peptide were only found in mice from groups 2 and 3, encoding the wild type STa peptide. In contrast to the antibody response, no STa specific IFN- γ was induced in mice from group 7, immunized twice with peptide only (Fig. 4c and d). A response just above the cut-off value was obtained in two other groups (groups 5 and 6) and a negative response was observed for group 4. These mice groups were primed

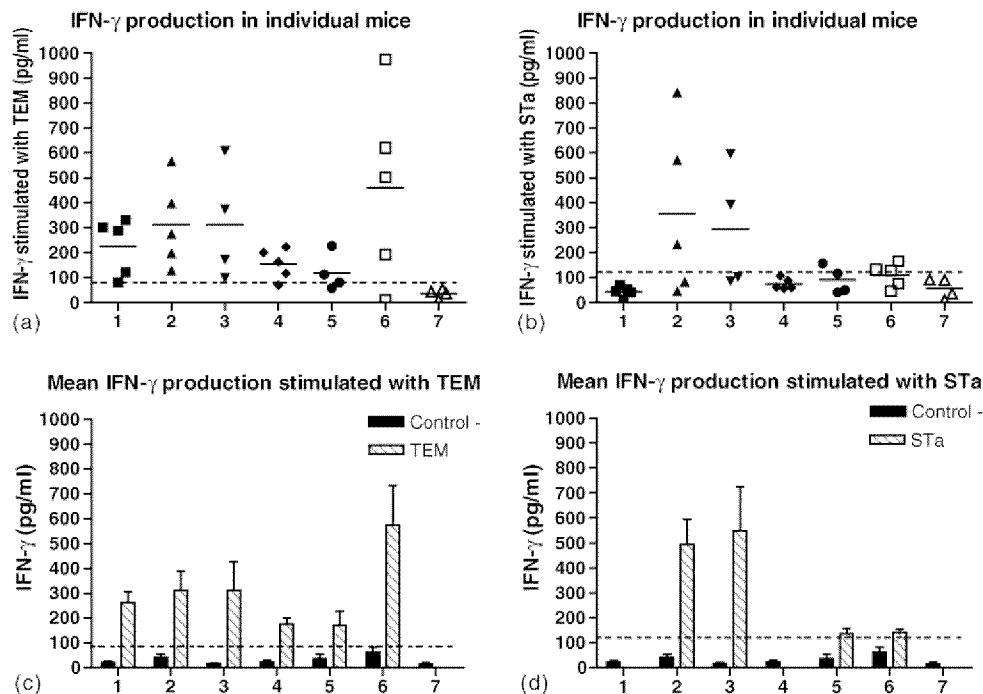


Fig. 4. IFN- γ production in 72 h culture supernatant from spleen cell of mice vaccinated with plasmid DNA encoding TEM195H (group 1), TEM216STa (group 2), TEM195STa (group 3), TEM195STa Cys6Ala (group 4), TEM195STa Cys17Ala (group 5), TEM195STa Cys6,17Ala (group 6), and control (group 7). This last group only received the STa peptide. Mice were sacrificed on day 147 and IFN- γ content was determined in supernatant from unstimulated cells (control) or from cells stimulated with TEM 10 μ g/ml (a and c) and with STa 10 pg/ml (b and d). Graphs a and b show IFN- γ production in individual mice. Horizontal bars represent the mean values for each group. Values obtained in group 7 were used to calculate a cut-off value for positivity (mean \pm 3 S.D., respectively, 93 and 110 pg/ml). These cut off were represented by the dotted line. Graphs c and d represent mean IFN- γ response of the animals with a positive response. IFN- γ levels (mean (pg/ml) \pm S.D.) in supernatants from non stimulated cells (control) or from stimulated cells.

with DNA plasmids containing the single-or double mutated STa.

3.6. Neutralization of the TEM-1 activity

The capacity to neutralize the enzymatic activity of β -lactamase in sera from plasmid DNA vaccinated mice was determined by measuring the initial hydrolysis rate of ni-

trocefin by enzyme-sera mixtures. Only sera, collected on day 63, from mice that showed a positive antibody response in ELISA were used. These sera were pooled for each group and diluted in the enzyme solution (2 μ g/ml). Anti-TEM-1 antibodies did not exert any inhibitory effect on the enzymatic activity at both two-fold or five-fold dilution, except for sera from group 2 that showed a weak inhibition (data not shown). Interestingly, mice from group

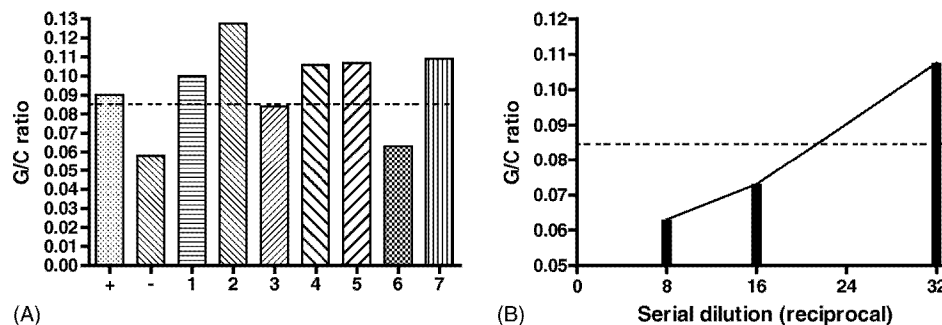


Fig. 5. (A) Neutralization assays of STa enterotoxin by sera (day 147) from animals primed with plasmid DNA encoding TEM195H (group 1), TEM216STa (group 2), TEM195STa (group 3), TEM195STa Cys6Ala (group 4), TEM195STa Cys17Ala (group 5), TEM195STa Cys6,17Ala (group 6), control (group 7) and boosted with STa. In each group, sera from immunised mice that showed positive STa antibody titres were pooled. These samples were diluted eight-fold in a STa solution. After a 16-h incubation at 4 $^{\circ}$ C, the suckling mouse assay was performed as described by Giannella [38]. Gut/carass weight ratios >0.085 are considered as positive for STa. The dotted line represents the toxicity threshold above which the samples are considered positive (not neutralized). (B) A serial dilution of sera from animals primed with TEM195STa Cys6-17Ala (group 6) and boosted with STa was made and residual enterotoxin activity was measured by the suckling mouse assay.

2 also displayed the highest IgG2a titres in ELISA against TEM-1.

3.7. Neutralization of the STa enterotoxicity

Sera from mice that scored positive in STa specific ELISA were pooled per group and the content in STa neutralizing antibody was determined by mixing with native STa. A 1:8 dilution of sera was prepared and the enterotoxicity of the mixture was determined by the suckling mouse assay. Only pooled sera of group 6 mice, primed with the V1J195STa Cys6,17Ala and boosted with the synthetic STa, exhibited toxin-neutralizing activity against native STa (Fig. 5A and B). This pool neutralised enterotoxicity of the native STa at dilution 1:8 and 1:16. The pool of group 3 mice primed with the V1J216STa sera was just at the cut-off value of the assay at dilution 1:8. The other pooled sera scored high above the cut-off value.

4. Discussion

DNA vaccination is an easy method for the generation of strong humoral and cellular immune responses, which offers several advantages as compared to vaccination with recombinant purified proteins. As there is no need for protein expression and purification, the product is easier to manufacture and in theory cheaper as well. Also, bacterial DNA has inherent adjuvant properties and through specific CpG containing oligonucleotide motifs, it can trigger the production of co-stimulatory molecules by professional antigen presenting cells through interactions with TLR-9. As a result of this, DNA vaccines have an intrinsic capacity to prime for polarized Th1-type CD4⁺ responses, polarization which is difficult to achieve with protein vaccines in classical adjuvants, such as alum. DNA vaccination induces a broader epitopic repertoire than vaccination with protein, and responses against both dominant and subdominant epitopes have been described in studies using plasmid DNA encoding antigens from *M. tuberculosis*, influenza and Sendai virus [23,24].

Here, we have demonstrated that DNA vaccination can also be used for the priming of antibody responses against a non-immunogenic peptide when the former is inserted as a peptide loop into a carrier protein, e.g. β -lactamase. STa enterotoxin from enterotoxigenic *E. coli* is poorly antigenic and non immunogenic in its native form. A potent vaccine inducing neutralizing antibodies against STa is not available for the moment but is badly needed. Indeed, in newborn calves and piglets, ETEC is a major cause of colibacillosis, characterized by profuse fluid diarrhoea, dehydration and even death [9,43] and as a result of this, ETEC is responsible for significant economic losses in agriculture. We genetically inserted the sequence encoding the STa oligopeptide (18 amino acids) into two different surface loops of the β -lactamase TEM-1 and, furthermore, three mutated STa sequences, in which one or two cysteine residues were replaced by alanines, were also

inserted in the TEM-1 molecule. These mutations disrupt one or two disulfide bridges and normally cause a complete loss of toxicity [16,30], but it is not known if these mutations also disrupt the immunogenicity and/or potential to induce neutralizing antibodies. In fact, Sanchez et al. have reported the construction of a detoxified STa fusion protein able to elicit antibodies against the STa toxin, but it was not assessed if these antibodies neutralized the biological activity of STa [16]. In the study presented here, BALB/c mice were vaccinated with the different plasmid constructs and we first analyzed antibody responses against the carrier protein. Although all plasmids had correct sequences and expressed the TEM-1 protein when tested in transiently transfected BHK-21 cells, the plasmid used in group 5, encoding the hybrid protein containing the cysteine17 mutated STa inserted in position 195 of TEM, did not induce any TEM-1 specific IgG1 or IgG2a antibodies, suggesting that this mutated STa affected the hydrophilic character of the TEM-1 loop resulting in disappearance of the B cell epitope. All five other plasmids induced TEM-1 specific antibodies, which were of comparable magnitude for IgG1 but significantly higher for IgG2a in mice from group 2, immunized with hybrid TEM-1 with the STa insert in position 216. This STa insertion in position 216 induced higher TEM-1 specific antibodies than the STa insertion in position 195 (group 3), which could indicate that insertions in loop B interfere less with the antibody production to the TEM-1 protein than insertions in loop A, probably because the immunodominant B cell epitope of TEM-1 is located on loop A. On the other hand, plasmid VJ216STa used in group 2 with the insert in loop B, induced the highest transient expression of TEM-1 in BHK-21 cells and this may also explain why highest anti-TEM-1 antibodies, capable to inhibit (albeit slightly) the enzymatic activity of the β -lactamase, were detected following vaccination with this plasmid. Th1 type immune responses against the TEM-1 protein, as measured by spleen cell IFN- γ secretion, could be detected following vaccination with the various plasmids. In contrast to the antibody responses (and to the in vitro transfection levels), TEM-1 specific IFN- γ titers were comparable in groups 2 and 3, in mice vaccinated with hybrid protein carrying the wild type STa insert at position 195 or 216, respectively. Strongest IFN- γ responses were found in mice from group 6, vaccinated with the hybrid carrying the double mutation of STa inserted into loop A.

With respect to STa specific responses, no antibodies could be measured after the three intramuscular DNA injections. As DNA vaccines are particularly effective as priming agent in prime-boost protocols [42], we decided to boost the plasmid-immunized mice with purified native peptide, which is not immunogenic in its native form. Following two injections of peptide, strong STa specific IgG1 and IgG2a antibodies could be detected by ELISA. Plasmid from group 6, encoding the double mutated STa insert in loop A of TEM-1 induced highest STa specific antibody titres, whereas peptide boosting of group 1 mice, vaccinated with DNA encoding the carrier TEM-1 protein only, induced no STa specific antibodies in

four mice out of five, confirming the lack of immunogenicity of the native peptide. Interestingly and somewhat in contradiction with findings in literature, mice vaccinated twice with peptide only, produced STa specific antibodies and these antibodies were more of IgG1 than of IgG2a isotype. In contrast, DNA primed mice produced STa specific antibodies of mixed IgG1/IgG2a isotype, indicative of the previous Th1 priming by the DNA vaccination. The fact that highest STa specific antibodies were found in group 6, which also had the highest IFN- γ production to the TEM-1 protein was an additional indication of priming of T-cell help through the carrier protein. Neutralization assays of the STa by the suckling mouse assay indirectly confirmed these ELISA findings as only pooled sera from group 6 mice (with the double mutation of STa inserted in position 195) were capable of neutralizing the toxicity of the enterotoxin. These results confirm a report by Pereira et al. [21] that the toxic activity of the STa is not needed to induce neutralizing antibodies. This may be of importance for future immunization strategies against stable enterotoxins in general. Therefore, it would be interesting to analyze the immunogenicity of plasmid DNA encoding the double mutation of STa in position 216, because insertion in loop B clearly gave the highest TEM-1 expression levels. On the other hand, insertion in loop A may be better to avoid competition between hapten and carrier B cell responses.

T-cell responses against the STa peptide were only induced in groups 2 and 3 mice, vaccinated with the wild type STa peptide. This was not surprising as the cysteine to alanine substitutions destroyed the amphipathic stretch between positions 9 and 14, that is indicative of a MHC class II restricted T-cell epitope on the STa toxin according to the TSites program [44]. This also indicates that the TEM-1 carrier protein rather than the STa peptide provided the necessary T-cell help for the enterotoxin specific antibody production.

In conclusion, our results show that immunization with plasmid DNA encoding hybrid hapten-carrier proteins, in casu β -lactamase with the *E. coli* STa insert, can be used as a priming stimulus to provide T-cell help in the antibody response against a non-immunogenic peptide and that the generation of this T-cell help is a critical factor.

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